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(54) Title: INHIBITION OF VIRAL REPLICATION

(57) Abstract

A method for preventing or treating infection of a mammal especially a human, by a virus, especially HIV-1, including administering a therapeutically effective amount of a sulfated and/or carboxylated chitosan derivative to the patient.

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INHIBITION OF VIRAL REPLICATION

Background of the Invention

The invention relates to methods of inhibition of viral replication, especially by therapeutic agents including polysaccharides.

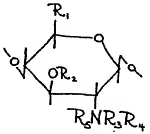
The escalating epidemic of the Acquired Immune Deficiency Syndrome (AIDS), caused by the Human Immunodeficiency Virus Type 1 (HIV-1), has created an urgent need to develop therapy to prevent or treat infections with this virus. The agent approved at present by the Federal Food and Drug Administration for the treatment of patients with AIDS is the nucleoside analog 3'-azido-3'-deoxythymidine (AZT). Dideoxcytidine (ddC) and dideoxyinosine (ddI) are being tested widely for such treatment.

Numerous sulfated polysaccharides have been shown to act as potent inhibitors of HIV-1 replication. Sulfated polysaccharides with anti-HIV-1 activity include: pentosan polysulfate, dextran sulfate, xylofuranan sulfate, ribofuranan sulfate, the bacterial sulfated glycosaminoglycan termed Org 31581 as well as chemically degraded heparin termed Org 31733, fucoidan sulfate, prunellin, lentinan sulfate, glycyrrhizin, heparin, carrageenans and mannan sulfate. One of these compounds, dextran sulfate, is currently undergoing clinical trials.

Summary of the Invention

The invention features a method for prevention of infection of a mammal, including humans by, or treatment of a mammal infected with, a virus, said method comprising the steps of providing an active agent in the form of a therapeutic polysaccharide comprising an ampholytic

(1→4)-2-amino-2-deoxy-β-D-glucan of polydispersed molecular weight comprising, at physiological pH, subunits of the structure



wherein

R_1 = $CH_2OSO_3^-$, CH_2OH, $CH_2OCH_2COO^-$, $CH_2OCH_2CHOHCH_2OH$, $CH_2OCH_2CH_2OH$, or COO^-;

R_2 = SO_3^-, H, CH_2COO^-, $CH_2CHOHCH_2OH$, or CH_2CH_2OH;

R_3 = SO_3^- or H;

R_4 = H, SO_3^-, $COCH_3$, CH_2COO^-, $CHCH_3CH_2CH_2COO^-$, $CH_2CHOHCH_2OH$, CH_2CH_2OH, N-ortho-carboxybenzyl, alkyl of from 1-6 carbon atoms, aldose or ketose residue attached via the aldehyde or keto carbon atom, or aromatic ring having at least one hydroxyl or carboxyl group; and

R_5 = no substituent or H^+,

provided that when R_4 is $COCH_3$, R_3 is H, and that the net charge on the polysaccharide is zero; and administering to the mammal a therapeutically effective amount of the polysaccharide in a pharmaceutically acceptable carrier.

In preferred embodiments, for between 10-85% of the subunits R_4 is $COCH_3$, for between 5-85% of the subunits R_4 is CH_2COO^-, and for between 0-85% of the subunits R_4 is SO_3^-. Preferably, when R_4 is H, R_3 is SO_3^-. The virus is preferably a retrovirus, especially HIV-1. The method of

administration is preferably oral, parenteral, or topical.

By polydispersed molecular weight is meant that the ratio of the weight average molecular weight and the number average molecular weight for a sample preparation is greater than 1 and preferably greater than 2.

By ampholytic is meant that the polysaccharide is an amphoteric electrolyte and can, at one time, behave as both an acid and a base. At physiological pH (approximately 7.4-7.6), substituent amino groups, having in general a pK_a of above 8.5, will usually be positively charged, and substituent carboxyl, or sulfate groups, having in general a pK_a of below 5.5, will usually be negatively charged. The protonation of the amino group at position 2 in subunits of the polysaccharide (i.e., whether R_5 = no substituent or H^+) will depend on the specific substituents at R_3 and R_4, which will influence the pK_a. The provision of one positively charged group and one negatively charged group only, as long as the net charge on the polysaccharide is zero, is sufficient to characterize the polysaccharide as ampholytic.

By therapeutic polysaccharide is meant a polysaccharide useful for the treatment of a disease or disorder. A therapeutically effective amount is that quantity which produces a significant physiological effect in the patient and is recognized by those of ordinary skill in the art to depend upon the size and weight of the mammal as well as other well known factors.

The polysaccharide as described above can be prepared from chitosan, itself a deacetylated derivative of chitin. The latter is an unbranched (1→4)-2-acetamido-2-deoxy-β-D-glucan that occurs in the exoskeleton of crustaceans and insects and in the cell wall of some bacteria and fungi.

Ampholytic sulfated or carboxylated derivatives of chitosan, as described above, are active inhibitors of HIV-1 replication with no significant toxic effects on cultured human or murine cells. They also have a wide spectrum of anti-retroviral activity, including inhibition of replication of oncogenic retroviruses such as Rauscher Murine Leukemia Virus (RLV). The compounds are highly soluble in aqueous solutions and are easily prepared according to conventional methods.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment thereof and from the claims.

Brief Description of the drawing

The Figure shows a typical preparation of N-carboxymethylchitosan-N,O-sulfate (NCMCS), representative of the class.

Description of the Preferred Embodiment

The sulfated chitosan derivative N-carboxymethyl-chitosan-N,O-sulfate (NCMCS), an amphoteric electrolyte or ampholyte, is an active inhibitor of the replication of
HIV-1 and Rauscher Murine Leukemia Virus (RLV) replication with no significant toxic effects on cultured human or murine cells. NCMCS can be used alone or in combination therapy with other anti-retroviral agents in the treatment of patients with AIDS. The zwitterionic effect occurring between the positive and negative centers of the molecule can enhance binding to the target virus or to virus-infected cells expressing viral antigens. The amphoteric nature of NCMCS is maintained even with a variation of the charge distribution on the polymer with different substitutients and varying degrees of substitution.

Structure of a typical preparation of NCMCS, representative of the class

NCMCS is a polydispersed molecular weight polysaccharide composed of (1→4)-2-amino-2-deoxy-β-D-glucan subunits. Referring to the Figure, in a typical preparation 66% of the subunits 12 contained an amino group 18 at the 2-position of the pyranose ring, 16% of the units 14 contained an acetamido group 20 at the 2-position, and 18% of the units 16 contained an N-substituted carboxymethyl group 22 at the 2-position. An undetermined fraction of units 12 and 16 also contained an N-substituted sulfate group 24 at the 2-position, and an undetermined fraction of all three units 12, 14, and 16, in addition, was substituted at the 3-position 26 or at the 6-position 28 with an O-substituted sulfate group. The individual subunits were arranged in random order in the polysaccharide.

At physiological pH any substituent carboxyl and/or sulfate group carried a negative charge, while the amino groups in the 2-position were generally protonated to carry a positive charge, depending on the N-substitutents in the specific subunit.

Preparation of NCMCS

Low molecular weight depolymerized chitosan from crab was obtained from Protan Labs, Redmond, Washington. N-Carboxymethylchitosan (NCMC) was prepared by a Schiff reaction of chitosan with glyoxylic acid (Muzzarelli et al., Carbohydrate Res. 107:199-204, 1982; Muzzarelli, Carbohydrate Polymers 8:1-21, 1988). Chitosan (80 gm) was added to 8.1 liters of 1.25% acetic acid with overhead stirring, and the chitosan gradually dissolved. After thirty minutes of stirring, the pH was 4 and the solution was clear. Glyoxylic acid solution (44 gm, 0.45 mole, in 200 ml water) was then added slowly and stirring continued for another two hours (pH 3.5). Sodium hydroxide (10N) was added in 25 ml aliquots to the solution with vigorous stirring, raising the pH to 5.

The total volume of sodium hydroxide required was approximately 250 ml.

Reduction of N-(carboxymethylidene) chitosan was effected by gradual addition of sodium borohydride (46 gm, 1.4 mole, in 250 ml water) with stirring over a 1 hour period. The pH rose to 6-7, the viscosity increased,and an insoluble gel and a soluble form of NCMC were produced. The gel was separated from the soluble NCMC by centrifugation, and the soluble form was dialyzed using a 1500 nominal molecular weight (MW) cutoff hollow fiber membrane (Vercellotti et al., New Developments in Industrial Polysaccharides, Crescenzi et al., eds., New York (1985), p. 125). Both the gel and the soluble form were separately precipitated by gradually pouring the NCMC into dry ethanol with stirring. The white precipitate formed was collected by centrifugation and dried to a white powder. Yield: 80 gm: NCMC gel - 85%, NCMC soluble - 15%.

The starting material for sulfation was an N-carboxymethylchitosan, as a dry powder from NCMC gel (the precipitate from NCMC soluble could have been used as well), with degree of substitution 16% N-acetyl; 18% N-carboxymethyl; and 66% free NH_2 as determined by ^{13}C nuclear magnetic resonance. NCMC gel (5 gm) was suspended in 50 ml of dry pyridine (Horton et al., Carbohydrate Res. 29:173, 1973). Chlorosulfonic acid (26 ml) was added carefully with a dropping funnel to dry pyridine (105 ml) in a closed flask fitted with a stirring motor, condenser, and drying tube. Ten ml of the complex were removed and stored in a dry flask for the second addition. The NCMC suspension was added to the pyridine-chlorosulfonic acid complex, and the mixture was heated in a water bath at 90-93°C for 4 hours. Stirring was then continued at room temperature.

After 18 hours the remaining 10 ml of pyridine-chlorosulfonic acid mixture were added to the NCMC mixture. The mixture was heated for 8 hours at 90°C in a water bath and then stirred for 14 hours. Water (100 ml) was added to destroy the excess chlorosulfonic acid. The mixture was brought to pH 8-9 with 10N sodium hydroxide, stirred for 24 hours, and allowed to settle for 1-2 hours. The supernatant was then siphoned off, and the mixture was added to dry ethanol with vigorous stirring. The precipitate was centrifuged, washed with dry ethanol, and collected. The NCMCS was redissolved in water, and the soluble portion was dialyzed with a 1500 nominal MW hollow fiber device. The aqueous solution was concentrated, precipitated with ethanol, and dried. Yield: 2.4 gm soluble NCMCS, 1.5 gm insoluble NCMCS.

<u>Analytical data for N-carboxymethylchitosan-N,O-sulfate</u>

<u>a) Elemental analysis of NCMCS</u>

Analytical determination of the molar ratios of carbon, nitrogen, hydrogen, and sulfur in the soluble NCMCS preparation were performed by standard techniques.

ELEMENT	RESULTS %	EXPERIMENTAL MOLAR RATIOS	CALCULATED MOLAR RATIOS
C	27.96	7.76	8.00
N	4.43	1.05	1.00
H	3.48	11.50	8.00-12.00
S	9.58	0.99	1.00

This analysis indicates approximately one sulfate group per sugar residue.

b) Molecular Weight Determination

Polystyrene sulfonate molecular weight standards ranging from 1,580 - 47,200 were used to calibrate three consecutive gel filtration columns in the Progel TSK - PWXL series: 2500, 3000, 4000, (Supelco, Bellefonte, PA) with 10% acetonitrile in 0.2M sodium chloride as solvent. The calibration equation, having a correlation coefficient of 0.98, was developed from ten runs of the molecular weight standards.

The molecular weight of the N-carboxymethylchitosan-N,O-sulfate (NCMCS) preparation was determined using the above system. Eighty-one and five-tenths per cent (81.5%) of the sample was in the inclusion volume. The weight average molecular weight (M_w) is 32,800 and the number average molecular weight (M_N) is 7,400. The ratio M_w/M_N is equal to 4.4, indicating a high degree of polydispersity.

The remaining 18.5% of the compound was excluded from the columns. The nominal exclusion value of the TSK column series used is 3 x 10^5; however, the molecular

weight ranges excluded will differ with the solute type due to the effective size of the molecules in solution. In addition, molecular folding and aggregation can cause a structure with the appearance of a larger mass per unit volume. The product was retained with a 1500 molecular weight cutoff hollow fiber ultrafilter.

c) Nuclear Magnetic Resonance Spectra

Nuclear magnetic resonance spectra (^{13}C) have signals for chemical shifts at 23 ppm (CH_3- of NHAc); 53 ppm (N-CH_2); 58 ppm (C-2); 68 ppm (C-2'); 73 ppm (C-6'); 78 ppm (C-3,5); 84 ppm (C-4); 107 ppm (C-1); 110 ppm (C-1'); 127 ppm (C=O of NHAc); 132 ppm (C=O of N-CH_2-CO_2H) indicative of O- and N- sulfation.

d) Infrared Spectra

Infrared spectra have characteristic N-carboxymethylchitosan backbone substituents and sulfate bands at 3600-3400 cm^{-1} (OH, NH); 1250 cm^{-1} (S=O); 1080-1000 cm^{-1}(C-O-C); and 810-800 cm^{-1} (C-O-S).

e) Other Specific Reactions

NCMCS gives a positive metachromatic reaction with toluidine blue, indicative of an O-sulfate ester or an N-sulfoamino group of a polysaccharide in a regular substitution pattern. With cetyl pyridinium chloride, NCMCS forms a precipitate that dissolves in 1M sodium chloride, indicative of an O-sulfate ester or an N-sulfoamino group on a polysaccharide. NCMCS is precipitable in 50% ethanol, and it is burned in the presence of sulfuric acid to an ash that is typical of sodium sulfate, indicating that a sodium salt of a sulfate ester was present in the original preparation. NCMCS does not give a positive ninhydrin reaction, indicating essentially complete N-substitution with N-acetyl, N-carboxymethyl, and/or N-sulfoamino.

Preparation of N-carboxymethylchitosan-N,O-sulfate for antiretroviral studies.

N-Carboxymethylchitosan-N,O-sulfate (NCMCS) prepared as described above, was obtained from V-LABS, INC. (Covington, LA). The material was dissolved in RPMI medium 1640 (Gibco, Gaithersburg, MD) at concentrations between 0.1-1.0 mg/ml and sterilized through a 45 μm Millipore filter. Stock solutions were stored at -20°C until use. Dilutions of NCMCS were made in complete RPMI medium 1640 or Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum, penicillin/streptomycin and L-glutamine. For enzyme kinetics experiments NCMCS stocks were prepared in sterile RNase-free water.

Inhibition of HIV-1 replication by NCMCS.

In cultured Jurkat cells, HIV-1 was inhibited very effectively by NCMCS as measured by reverse transcriptase activity in tissue culture supernatants. About 1 x 10^5 Jurkat cells (human T cells) per well in six well dishes were incubated with different concentrations of NCMCS for 24 h. Subsequently, a standardized inoculum of HIV-1 (2000 reverse transcriptase cpm units/well) was added to the cells which were incubated in the continued presence of the appropriate concentrations of drug. After 5 days, production of HIV-1 was determined by reverse transcriptase assays using poly$(A)_n$·oligo(dT) as template primer and ^{3}H-TTP as substrate as previously described (Roy-Burman et al., J. Virol. 19:1107-1110, 1976). Simultaneously, the cytotoxicity of NCMCS was determined by trypan blue exclusion.

NCMCS inhibited HIV-1 with an inhibitory concentration $_{50}$(IC_{50}) of 7 μg/ml. No significant cytotoxicity was noted over a wide concentration range,

including a concentration of 0.17 mg/ml. Thus, the therapeutic index of NCMCS remains undetermined because of a lack of cytotoxicity.

Inhibition of Rauscher Leukemia Virus by NCMCS.

To test the activity of NCMCS against a murine leukemia virus, the modification of the XC plaque assay (Rowe et al., Virology 42:1136-1139, 1970) was used with Rauscher murine leukemia virus (RLV), strain RVB3, a virus complex that causes a rapidly fatal erythroid disease in mice and which has been employed for the rapid in vivo analysis of candidate antiretroviral agents (Ruprecht, Intervirology 30 (suppl. 1):2-11, 1989), as the test virus. After pretreatment of murine SC-1 fibroblasts for 24 h with various concentrations of the NCMCS, the cells were infected with RLV particles in the presence of 8 μg/ml polybrene. Different virus inocula were used, namely 5.5 x 10^2 or 5.5 x 10^3 plaque-forming units per 1 x 10^5 SC-1 cells per well, using six well dishes. NCMCS was present throughout the duration of the experiment until the SC-1 cells were UV-killed 5 days after virus infection. Expression of retroviral envelope was assessed by the addition of untreated XC indicator cells (5.0 x 10^5 per well), and the number of syncytia was determined 72 h later. In parallel, cell toxicity at each concentration was determined in triplicate by trypan blue exclusion.

NCMCS was very active in this assay; RLV-induced plaques were inhibited at an IC_{50} of 0.4 μg/ml. Again, at the concentrations that were tested, no cytotoxicity was detected in the SC-1 fibroblasts. This lack of toxicity prevented the estimation of a therapeutic window.

Inhibition of HIV Reverse Transcriptase by NCMCS.

The kind of inhibition of HIV-1 reverse transcriptase by NCMCS was determined by standard enzyme kinetics. Reactions were performed in 50 μl of 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 80 mM KCl, 1 mM dithiothreitol, 1 μM TTP, 250 μCi/ml ^{3}H-TTP (New England Nuclear, specific activity 86.3 Ci/mmol), poly(A)$_n$·oligo(dT), 1 unit of human placental RNase inhibitor (RNasin, Promega Biotech) and NCMCS at different concentrations. Purified recombinant HIV-1 reverse transcriptase was added last to the reaction mixture, and the reactions were run at 37°C for 1 h. The reaction mixtures were spotted directly onto Whatman DE81 filters; washed extensively in 0.3M NaCl, 0.03M Na citrate, pH 7.0, and finally in ethanol. After the filters were dried, the degree of ^{3}H-TTP incorporation was measured by liquid scintillation spectroscopy. All analyses were carried out in the linear phase of incorporation of ^{3}H-TTP, using poly (A)$_n$·oligo(dT) as template/primer. When the concentration of poly(A)$_n$·oligo(dT) and the concentration of the inhibitor were varied in the standard enzyme assay, NCMCS was observed to competitively inhibit the reverse transcriptase of HIV-1.

While not being bound to any theory, it appears that in addition to inhibiting retroviral reverse transcriptase, NCMCS may inhibit retroviral adsorption to the cellular receptor due to its ampholytic nature.

Presence of HIV-1 Proteins in NCMCS-treated Cells

To confirm the data obtained in Jurkat cells by measuring reverse transcriptase activity in tissue culture supernatants, the presence of HIV-1 proteins in NCMCS-treated cells was examined by immunoprecipitation. Jurkat cells pretreated with NCMCS were incubated with fully infectious HIV-1 as described above. After 7 days,

the cells were washed extensively with phosphate buffered saline (PBS), pH 7.2, and 1.5 x 10^6 cells were labelled for 14 h with 200 μCi/ml of ^{35}S-cysteine at a specific activity of 1,000 Ci/mmol in cysteine-free RPMI medium 1640 supplemented with 10% fetal calf serum, penicillin/streptomycin and L-glutamine. The synthesis of HIV-1 specific proteins was monitored by immunoprecipitation analysis with an AIDS patient serum, followed by 7% SDS-polyacrylamide gel electrophoresis and autoradiography. A dose-dependent decrease of HIV-1-specific protein synthesis with increasing concentrations of the inhibitor was observed. At a concentration of 0.17 mg/ml, no viral proteins could be detected. As in the previous experiments, no cytotoxic effects of NCMCS could be noted at any NCMCS concentration.

<u>Use</u>

As NCMCS is an active inhibitor of HIV-1 replication with no significant toxic effects on cultured human cells, NCMCS can be used therapeutically in the prevention of or treatment of Acquired Immune Deficiency Syndrome, or AIDS. NCMCS may be administered orally, parenterally, or topically by routine methods in pharmaceutically acceptable inert carrier substances. Optimal dosages and modes of administration can readily be determined by conventional protocols.

In addition, the compounds may be adminstered prophylactically or after infection to treat infection by any virus, especially any retrovirus, including oncogenic retroviruses such as RLV. For example, the compounds may be administered as a vaccine adjuvant to enhance the production of antibodies. The compounds may also be coadministered with other therapeutic agents to aid in the delivery of the therapeutic agent and to enhance the effectiveness of the agent. The compounds may be used to coat or microencapsulate the vaccine or therapeutic agent

or may be coadministered orally, parenterally, or topically. NCMCS may also be used in combination therapy with antiviral agents with different mechanisms of inhibition and non-overlapping toxicity.

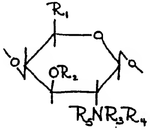
Other embodiments are within the following claims. Any sulfated and/or carboxylated derivative of chitosan having the water solubility and amphoteric properties of NCMCS is suitable as a therapeutic polysaccharide in the method of the invention. For example, some subunits may contain an O-substituted carboxymethyl group at position 6 or position 3 of the pyranose ring and no sulfate substituents, or the carbon atom itself at position 6 may be substituted by a carboxyl group. Other charged substituents at position 2 may include N-substituted carboxyalkyl and N-ortho-carboxybenzyl residues, and aromatic rings having at least one carboxyl group. Non-charged substituents, including hydroxyalkyl, aldose or ketose residues attached via the aldehyde or keto carbon atom, and aromatic rings having at least one hydroxyl group, may be N-substituted at position 2, and hydroxyalkyl residues may be O-substituted at position 6, to enhance the solubility of the polysaccharide.

What is claimed is:

1. A method for prevention of infection of a mammal by, or treatment of a mammal infected with, a virus, said method comprising the steps of

providing an active agent in the form of a therapeutic polysaccharide comprising

a polydispersed molecular weight, ampholytic (1→4)-2-amino-2-deoxy-β-D-glucan comprising, at physiological pH, subunits of the structure



wherein

R_1 = $CH_2OSO_3^-$, CH_2OH, $CH_2OCH_2COO^-$, $CH_2OCH_2CHOHCH_2OH$, $CH_2OCH_2CH_2OH$, or COOH;

R_2 = SO_3^-, H, CH_2COO^-, $CH_2CHOHCH_2OH$, or CH_2CH_2OH;

R_3 = SO_3^- or H;

R_4 = H, SO_3^-, $COCH_3$, CH_2COO^-, $CHCH_3CH_2CH_2COO^-$, $CH_2CHOHCH_2OH$, CH_2CH_2OH, N-ortho-carboxybenzyl, alkyl of from 1-6 carbon atoms, aldose or ketose residue attached via the aldehyde or keto carbon atom, or aromatic ring having at least one hydroxyl or carboxyl group; and

R_5 = no substituent or H^+,

provided that when R_4 is $COCH_3$, R_3 is H, and that the net charge on said polysaccharide is zero; and

administering to the mammal a therapeutically effective amount of said polysaccharide in a pharmaceutically acceptable carrier.

2. The method of claim 1 wherein for between 0-85% of the subunits of said therapeutic polysaccharide, R_4 is $COCH_3$.

3. The method of claim 1 wherein for between 5-85% of the subunits of said therapeutic polysaccharide, R_4 is CH_2COO^-.

4. The method of claim 1 wherein for between 5-85% of the subunits of said therapeutic polysaccharide, R_4 is SO_3^-.

5. The method of claim 1 wherein for between 5-85% of the subunits of said therapeutic polysaccharide, R_1 is COOH.

6. The method of claim 1 wherein when R_4 is H, R_3 is SO_3^-.

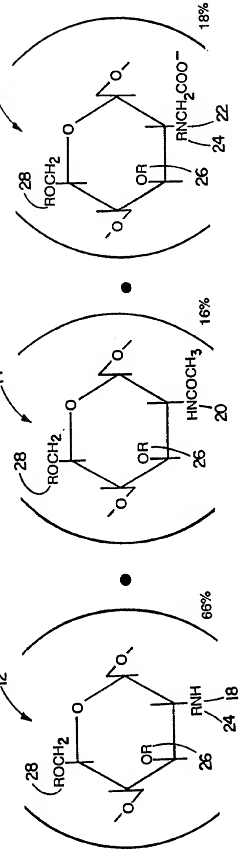
7. The method of claim 1 wherein when R_3 is H, R_4 is SO_3^-.

8. The method of claim 1 wherein when R_3 is H, R_1 is SO_3^-.

9. The method of claim 1 wherein said virus is a retrovirus.

10. The method of claim 1 wherein said retrovirus is HIV-1.

11. The method of claim 1 wherein said administering is oral, parenteral, or topical.



WHERE R=SO_3^- OR H

FIGURE

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07232

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/725
U.S. CL. 514/55

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/55 ; 536/20

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,840,941 (UENO) 20 JUNE 1989; See the Abstract; column 11, line 60; example 6 and the table in example 11.	1-11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 December 1991	21 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
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